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REMARKS/ARGUMENTS

Claims 1-4, 9-13, and 15-30 are pending in the application. Claims 1-4, 10-13, 15-17, 19, 23, and 24 were allowed by the Examiner. Pending claims 2, 4 and 28 have been amended to correct minor typographical errors.

Claim Rejection Under 35 U.S.C. § 112

The Examiner has rejected claims 9 and 25-30 under 35 U.S.C. § 112 as vague and indefinite because of the phrases surrounding the *Citrus aurantium* extracts. The claims have been amended as suggested by the Examiner to recite "an extract of *Citrus aurantium* containing synephrine therein or other natural source of synephrine."

Claim Rejection Under 35 § 102

The Examiner has rejected claims 25-30 under 35 U.S.C. § 102(e) as anticipated by Murad *et al.* (US 6,207,694), with evidence provided by Pierce (Am. Pharm. Assoc. - Practical Guide to Natural Medicaines, 1999). Specifically, the Examiner suggests that Murad teaches compositions of saw palmetto and *Citrus aurantium*. Pierce is provided as evidence that saw palmetto is also known as *Serenoa repens* and *Serenoa serrulata*.

The Murad reference does not anticipate the instant invention. Claim 25 reads in part "wherein the sympathomimetic agent is an extract of *Citrus aurantium* containing synephrine therein." Murad teaches only the use of essential oils derived from *Citrus aurantium* (see Examples 2, 5, and 6), not an extract. There is no disclosure in the reference of an extract of Citrus that contains synephrine. Furthermore, synephrine is a water soluble constituent (see Kusu et al. (1996) *Anal. Biochem.*, where an aqueous extraction process was performed to quantify synephrine from different *Citrus* species (Exhibit A); Ibrahim et al. (1984) *Anal. Chem.*, where synephrine is recovered in urine (Exhibit B); and Stewart et al. (1964) *J. Biol. Chem.*, where synephrine is recovered from Citrus leaves using alcohol extraction). Claim 25 reads in

part "wherein the sympathomimetic agent is at a concentration between 5-5,000 mg" and wherein the sympathomimetic agent from *Citrus* is a "source" of synephrine. The essential oil of *Citrus* species will not contain appreciable quantities of the water soluble compound. The highest concentration exemplified is 0.5% by weight of the formulation of an essential oil *blend* (Example 2) derived from eight species, only two of which are *Citrus aurantium*. The range of dosages of the composition are provided as from "about 1 mg to 20,000 mg, more preferably about 2,000 to 16,000 mg and most preferably about 6,000 to 10,000 mg of the active components" (column 9, lines 30-38). The Murad patent thus does not anticipate the pending claims.

Applicant respectfully requests that a timely Notice of Allowance be issued in this case.

Respectfully submitted,

By Madeline Johnston by express permission Susanne Hollinger Reg. 51,811

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Exhibit A

ANALYTICAL BIOCHEMISTRY 235, 191–194 (1996)
ARTICLE NO. 0111

Determination of Synephrine Enantiomers in Food and Conjugated Synephrine in Urine by High-Performance Liquid Chromatography with Electrochemical Detection

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Received September 18, 1995

Determination of synephrine enantiomers was made with HPLC with electrochemical detection using a chiral ligand-exchange column (Sumichiral OA-6000). The calibration curve for each enantiomer showed good linearity (r = 0.999) at 1.0–500 pmol injected with a detection limit of 1.0 pmol (signal-to-noise ratio (S/N) = 3). Relative standard deviation (RSD) was 1.8% at 50 pmol *d*-synephrine and 1.6% at 50 pmol *l*-synephrine. The contents of synephrine enantiomer in food such as *Citrus unshiu* fruit, orange juice, and marmalade were determined. The present method was also used to determine conjugated synephrine enantiomers in urine following the ingestion of *C. unshiu* pulp. *l*-Synephrine in fruit was converted to the conjugated form of synephrine, and *l*-synephrine underwent chiral inversion to *d*-synephrine *in vivo*.

Synephrine is a synthetic sympathomimetic drug that exhibits various types of pharmacological activity such as vasoconstriction, blood pressure elevation, and bronchial muscle relaxation (1). It is found in crude drugs (2), citrus plants (3), and human urine after the ingestion of orange fruit; Stewart *et al.* isolated *l*-synephrine from the leaves and juice of citrus plants (3). Synephrine is a chiral compound. Although synephrine enantiomers exhibit different pharmacological activities (4), the determination of synephrine enantiomers in urine has so far not been made (5, 6). Some drugs undergo chiral inversion *in vivo* (7). However, whether chiral inversion of synephrine occurs in the human body remains to be clarified.

In a previous study, synephrine enantiomers were determined by HPLC on a Sumichiral OA-5000 chiral ligand-exchange column using an electrochemical detector with high sensitivity (8). In this study, determination of synephrine enantiomers in food originating from citrus plants and in human urine after ingestion of *Citrus unshiu* pulp was carried out with HPLC and a different kind of chiral ligand-exchange column, Sumichiral OA-6000. With this column, separation was possible not only of *d*- and *l*-synephrine but also of *d*-synephrine and *N*-methyltyramine, the latter often present in citrus plants.

MATERIALS AND METHODS

Reagents

dl-Synephrine was purchased from Sigma Chemical Co. (St. Louis, MO), *l*-phenylephrine was from Tokyo Kasei Kogyo Co. (Tokyo, Japan), copper(II) acetate was from E. Merck (Darmstadt, Germany), and ammonium acetate, ethyl acetate, and *n*-butanol were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Apparatus

The HPLC system consisted of a Jasco 880-PU pump (Jasco, Tokyo, Japan), an 8125 injector fitted with a 5-μl injection loop (Reodyne, Cotati, U.S.A.), a Sumichiral OA-6000 column (150 × 4.6 mm i.d., Sumika Chemical Analysis Service, Osaka, Japan), and an EDP-1 electrochemical detector (Kotaki, Japan). The Extrelut column was purchased from E. Merck.

Sample Preparation for Foods

Juice. Water and *l*-phenylephrine as the internal standard were added to 1 ml orange juice to give a 25-ml sample solution.

C. unshiu fruit and marmalade. C. unshiu fruit was divided into exocarp, mesocarp, endocarp, and sarcocarp portions, each of which was homogenized and extracted with water under ultrasonication at room temperature for 10 min. After centrifugation, the pre-

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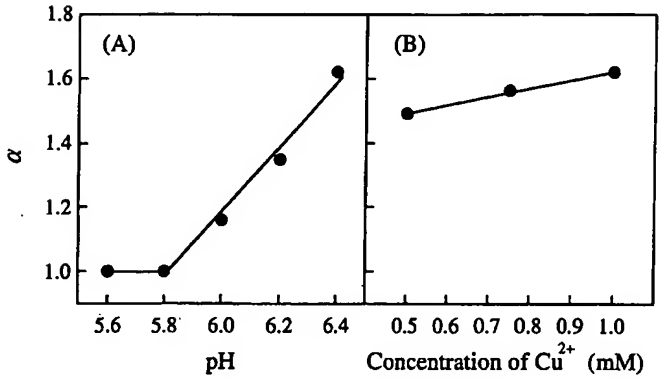

FIG. 1. Effect of pH and copper(II) ion concentration in the mobile phase on the elution of synephrine enantiomers. HPLC conditions: (A) mobile phase, water containing 1 mM copper(II) acetate and 20 mM ammonium acetate (pH 5.6–6.4); column, Sumichiral OA-6000 (150 × 4.6 mm i.d.); flow rate, 1.5 ml/min; applied potential, 1.0 V vs Ag/AgCl; (B) mobile phase, water containing 0.5–1 mM copper(II) acetate and 20 mM ammonium acetate (pH 6.4); other HPLC conditions the same as in (A).

cipitate was reextracted in the same manner and the supernatants were combined. *l*-Phenylephrine and water were added to the supernatant thus obtained to give a certain volume of sample solution. Marmalade was homogenized with water at 50°C and extracted under ultrasonication at room temperature for 10 min. The procedure was the same for *C. unshiu* fruit.

All sample solutions were filtered through a 0.45-μm membrane filter before the sample solution was injected into the HPLC system.

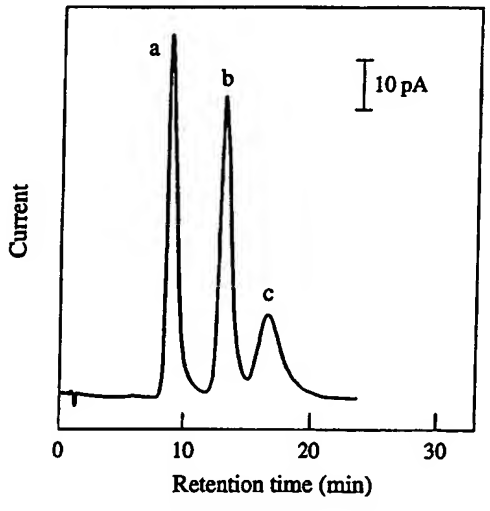

FIG. 2. Chromatogram of a standard mixture of *dl*-synephrine and *l*-phenylephrine. Peaks: (a) *l*-synephrine (100 pmol); (b) *d*-synephrine (50 pmol); (c) *l*-phenylephrine (50 pmol); HPLC conditions: aqueous mobile phase containing 1 mM copper(II) acetate and 20 mM ammonium acetate (pH 6.4); column, Sumichiral OA-6000 (150 × 4.6 mm i.d., 5 μm); flow rate 1.5 ml/min; applied potential 1.0 V vs Ag/AgCl.

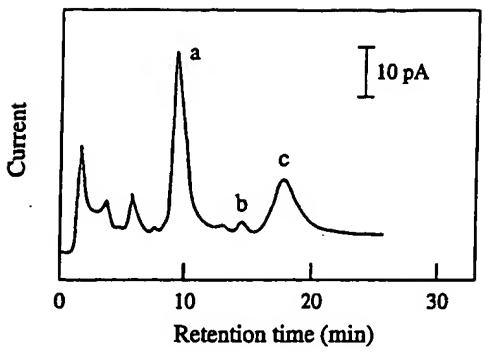

FIG. 3. Chromatogram of a sample solution extracted from orange juice. Peaks and HPLC conditions as in Fig. 2.

Preparation of Urine Sample

Subjects. Three young adults in good physical health served as the subjects in this study. Subject A was a male, age 25 and weighing 65 kg. Subject B was a male, age 23 and weighing 62 kg. Subject C was a male, age 24 and weighing 90 kg.

Urine collection. After being on a plant-free and continuously controlled component diet for 3 days, each subject provided blank urine. All three then consumed *C. unshiu* pulp containing sarcocarp and endocarp. Urine was collected 11 times for 24 h. All samples were adjusted to pH 1–2 with 6N HCl and stored at −30°C until use.

Sample preparation. For hydrolysis of the urine samples, each was heated at 100°C for 15 min. Under this hydrolysis condition, decomposition of *l*-synephrine in *C. unshiu* sample solution was not observed. The acid-hydrolyzed urine was then adjusted to pH

TABLE 1

Seasonal Comparison of Synephrine Enatiomers in *Citrus unshiu* Fruit

Part of *C. unshiu* fruit	Content (mg/sample 100 g)	
	d-Synephrine	*l*-Synephrine
Pericarp		
Exocarp		
Unripe	—[a]	84
Ripe	—	58
Mesocarp		
Unripe	—	256
Ripe	—	115
Endocarp		
Unripe	—	135
Ripe	—	78
Sarcocarp		
Unripe	—	4
Ripe		3

[a] Not detected (<1 mg/sample 100 g).

TABLE 2

Content of Synephrine Enatiomers in Food from Citrus Plants

Sample	Content (mg/sample 100 g)		Recovery (%)[a]	RSD (%)[b]
	d-Synephrine	*l*-Synephrine		
Orange juice 1[c]	0.11	2.33	95.0	1.9
Orange juice 2[d]	0.13	3.55	94.0	2.3
Marmalade	0.36	12.1	101.5	2.2

[a] 2 nmol *dl*-synephrine spiked to 1 g sample.
[b] $n = 5$.
[c] Made from valencia orange.
[d] Made from unshiu mikan.

9. One milliliter of the solution was applied to the Extrelut column. After 15 min, synephrine enantiomers were eluted with 5 ml ethyl acetate containing 2% *n*-butanol. The eluate was evaporated. This procedure was carried out twice, the residue was then dissolved in 225 μl of water, and 25 μl of 0.2 mM *l*-phenylephrine as the internal standard solution was added. Five-microliter aliquots of the solution were injected into the HPLC system.

RESULTS AND DISCUSSION

Synephrine Enantiomer Determination

With a ligand-exchange HPLC column, mobile-phase conditions such as solvent, pH, and metal ion concentration are significant factors. The stationary phase of the Sumichiral OA-6000 column consists of a coordination compound of copper(II) ion and the chiral ligand (*R,R*)-tartaric acid mono-(*R*)-1-(α-naphthyl)ethylamide (9). Separation is based on equilibrium of the formation of copper(II) complexes with solutes, such as synephrine enantiomers. To optimize conditions, pH and copper(II) ion concentration in the mobile phase were adjusted for enantiomer separation. Figure 1 shows the effects of pH and copper(II) ion concentration in the mobile phase on the separation factor (α) of the synephrine enantiomers. On the chromatogram obtained below pH 5.8, the separation of synephrine enantiomers was not completed; that is, $\alpha = 1$. Above pH 5.8, α increased with pH. However, a precipitate formed in the mobile phase at pH 6.5 or higher. Consequently, the pH of mobile phase could not be made higher than 6.4. α also increased with copper(II) ion concentration. The optimum concentrations of the mobile phase were thus 1 mM copper(II) acetate and 20 mM ammonium acetate in aqueous solution (pH 6.4). Synephrine was oxidized at above 0.8 V vs Ag/AgCl. The detection potential of 1.0 V vs Ag/AgCl was determined based on the hydrodynamic voltammogram of synephrine (10). Figure 2 shows a typical chromatogram of a standard mixture of *dl*-synephrine and *l*-phenylephrine as the internal standard compound. When the flow rate of the mobile phase was 1.5 ml/min, the retention times of *l*-synephrine, *d*-synephrine, and *l*-phenylephrine were 9.0, 13.5, and 17.0 min, respectively. α and resolution (*R*) for both synephrine enantiomers were 1.62 and 2.25, respectively. Peak current ratios relative to the internal standard on the chromatograms for solutions containing the internal standard and synephrine enantiomers were plotted against the concentrations of synephrine enantiomers. A good linear relationship between concentration and peak current ratio was found at 1.0–500 pmol injected with a correlation coefficient of 0.999 for each synephrine enantiomer. RSD was 1.8% at 50 pmol *d*-synephrine ($n = 10$) and 1.6% at 50 pmol *l*-synephrine ($n = 10$). The detection limit (S/N = 3) of each synephrine enantiomer was 1.0 pmol per injection. This column is thus shown to be superior to Sumichiral OA-5000 in the resolution of synephrine enantiomers.

Synephrine Enantiomers in Food Originating from Citrus Plants

The peelings of citrus plant fruit contain *l*-synephrine only (10). However, the synephrine enantiomer content in juice, jam, and the pulp of citrus plant fruit has not been previously determined and was thus done in this study. Figure 3 shows a chromatogram of a sample solution extracted from orange juice. There was no interference with the peaks of synephrine enantiomers of *C. unshiu* fruit. This fruit consists of exocarp, mesocarp, endocarp, and sarcocarp. The latter two are usually ingested as pulp. Table 1 shows the contents of synephrine enantiomers for each *C. unshiu* fruit component. *d*-Synephrine was not detected and thus

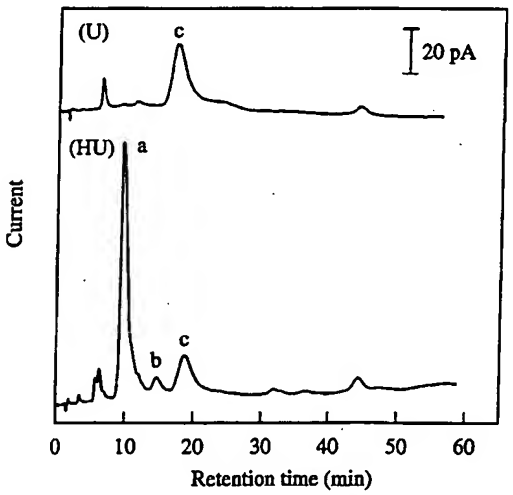

FIG. 4. Chromatogram of a sample solution extracted from urine (U) and acid-hydrolyzed urine (HU). The urine sample was obtained from a healthy subject (male, 25 years old, 65 kg) after ingestion of 240 g *C. unshiu* pulp. Peaks and HPLC conditions as in Fig. 2.

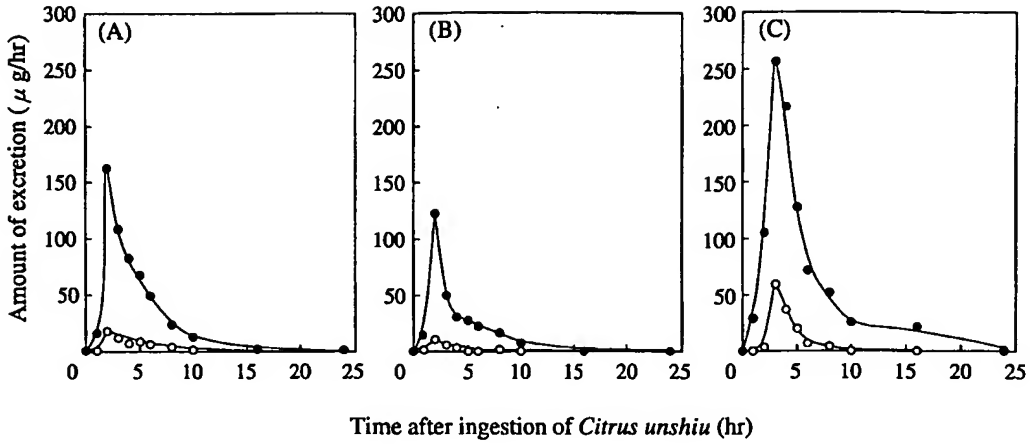

FIG. 5. Amounts of excreted conjugated synephrine enantiomers (O, *d*-synephrine; ●, *l*-synephrine) after ingestion of *C. unshiu* pulp. The urine sample was collected from subject A after ingestion of 240 g *C. unshiu* pulp (A), subject B after ingestion of 230 g *C. unshiu* pulp (B), and subject C after ingestion of 275 g *C. unshiu* pulp (C).

was present at less than 0.001% in all cases. Table 2 shows the contents of synephrine enantiomers in juice and marmalade originating from citrus plants. Recovery of 2nmol *dl*-synephrine spiked to 1 g sample was 94 to 105%. *d*-Synephrine as well as *l*-synephrine were detected in orange juice and marmalade. *d*-Synephrine may thus possibly be formed during production using citrus fruits.

Monitoring Synephrine Enantiomers in Urine after Ingestion of C. unshiu Pulp

Urine samples from subjects A, B, and C after the ingestion of *C. unshiu* pulp were analyzed. All synephrine excreted in urine is in the conjugated form (11) and thus the conjugated synephrine in urine in this study was refluxed at 100°C for 15 min at pH 1–2. This treatment did not cause chiral inversion. Figure 4 shows the chromatogram of a sample solution extracted from urine (U) and acid-hydrolyzed urine (HU). There was no interference from the urine extract with the peaks of the synephrine enantiomers. *d*-Synephrine and *l*-synephrine were detected in acid-hydrolyzed urine but not in urine. Chromatograms for urine and acid-hydrolyzed urine obtained before ingestion were the same as those in Fig. 4 (U). Recovery of 0.1 μmol *d*- and *l*-synephrine spiked to 5 ml urine was 99.8%, thus clearly indicating conjugated synephrine enantiomers to be excreted from urine after the ingestion of a *C. unshiu* pulp.

Figure 5 shows the time courses of excretion of conjugated synephrine enantiomers in the urine after ingestion of *C. unshiu* pulp. Maximum excretion of synephrine enantiomers was observed 2–3 h later, and synephrine enantiomers disappeared from the urine at 16 h following ingestion. *C. unshiu* pulp contains *l*-synephrine only, and thus 20–50% of *l*-synephrine in ingested *C. unshiu* pulp was excreted as the conjugated form in urine and about 10% of conjugated synephrine excreted in urine was conjugated *d*-synephrine. Chiral inversion of *l*-synephrine would thus appear to occur *in vivo*. HPLC with electrochemical detection using a chiral ligand-exchange column is shown by the present study to be useful for elucidating the metabolism of *l*-synephrine.

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Exhibit B

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RECEIVED for review February 21, 1984. Accepted April 19, 1984. This research was supported in part by Grants DA02277, CA32389, HL29476, DA01696, DA02088, DA02538, DA00053, and DA00065 from the National Institutes of Health.

Quantitative Measurement of Octopamines and Synephrines in Urine Using Capillary Column Gas Chromatography Negative Ion Chemical Ionization Mass Spectrometry

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The isomeric octopamines and synephrines were measured in urine by a new assay which combines ion-exchange chromatography, capillary column gas chromatography, and electron capture negative ion chemical ionization mass spectrometry. Deuterium labeled analogues of each compound were added to urine containing 1 mg of creatinine and the acid hydrolysate was subjected twice to cation exchange chromatography. The resultant amine fraction was derivatized with pentafluoropropionic anhydride and, under electron capture negative chemical ionization conditions, the PFP derivatives gave M^- and $(M - HF)^-$ ions which were sufficiently abundant to be suitable for selected ion monitoring. The limit of detection was approximately 100 pg mg^{-1} creatinine. In 10 normal adults, the concentrations of *o*-, *m*-, and *p*-octopamine and *o*-, *m*- and *p*-synephrine were, respectively, 0.6 (±0.2), 2.1 (±0.8), 25 (±19), ND (<0.1), 1.8 (±0.7), and 16 (±10) ng mg^{-1} creatinine. The occurrence of *o*- and *m*-octopamine and *m*-synephrine in human tissue or fluid has not been reported previously.

The development of a radiochemical enzyme assay for the quantitative determination of *p*-octopamine (*1*, *2*) led to its discovery in several invertebrate nerve systems and in sympathetically innervated mammalian organs. However, it was subsequently discovered that the method was not specific for *p*-octopamine because the meta and para isomers were not resolved. The use of a modified method showed that both *m*- and *p*-octopamine were present in rat salivary gland (*3*) and brain (*4*). The radiochemical enzyme assay depends upon norepinephrine *N*-methyltransferase, which can accept all three positional isomers of octopamine as substrates with varying efficacy. The resultant products, the corresponding synephrines, are also substrates for the enzyme (*5*, *6*), and consequently, any one or more of these amines could have been detected and quantified as *p*-octopamine by the unmodified assay.

Gas chromatography/mass spectrometry (GC/MS) techniques permit the unequivocal identification and quantitative determination of the three isomeric octopamines and three isomeric synephrines and there is now conclusive evidence for the natural occurrence in mammalian tissue of *o*-octopamine (*7*), *m*-octopamine (*3*, *4*, *8*), *m*-synephrine (*9*, *10*), and *p*-synephrine (*10*). Our initial attempts to determine naturally occurring isomeric octopamines and synephrines in mammalian urine by electron impact GC (packed column) MS methods were unsuccessful because the concentrations of these amines in most of the samples were below the lower limits of detection (50–100 ng mg^{-1} creatinine) of the method. For this reason, we turned to electron capture negative chemical ionization (NCI) GC/MS because of its reported 10–100-fold increase in sensitivity over electron impact methods (*11*, *12*). The use of an ion-exchange resin (to separate and concentrate very small amounts of amines for analysis by NCI GC/MS) together with an "ultrabond" capillary column has enabled us to demonstrate the natural occurrence of *o*-, *m*-, and *p*-octopamine and *m*- and *p*-synephrine in normal human urine. *m*-Synephrine and *o*- and *m*-octopamine have not been identified previously in any human tissue or fluid.

EXPERIMENTAL SECTION

Reagents. These were obtained from the following sources: pentafluoropropionic anhydride (PFPA), Pierce Chemical Co. (Rockford, IL); *m*-octopamine hydrochloride, Interchim (Montlucon, France); *m*-synephrine, Sterling Chemical Co. (New York, NY); phenylethanolamine hydrochloride, *p*-synephrine, epinine hydrochloride, *p*-octopamine hydrochloride, and epinephrine bitartrate, Regis Chemical Co. (Morton Grove, IL); dopamine hydrochloride and metanephrine hydrochloride, Sigma Chemical Co. (St. Louis, MO); norepinephrine hydrochloride, 3-methoxytyramine hydrochloride, and normetanephrine hydrochloride, Calbiochem (Los Angeles, CA); *m*-tyramine hydrochloride, Vega Fox Biochemicals (Tucson, AZ); *p*-tyramine, Aldrich Chemical Co. (Milwaukee, WI); strong cation-exchange resin (AG 50W-X2, 100/200 mesh, H^+ form), Bio-Rad (Richmond, CA); *o*-octopamine-α,α'-d_2-β-d_1 hydrochloride, Merck, Sharp & Dohme (Montreal, Canada).

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Table I. Composition of Deuterated Amines (%)

compound	d_0	d_1	d_2	d_3	d_4	d_5
o-octopamine-d_3	0.1	1.0	12.2	86.1	0.7	
m-octopamine-d_3	0.1	1.1	15.3	81.5	2.0	
p-octopamine-d_4	0.0	0.0	0.7	6.8	85.8	6.6
o-synephrine-d_3	1.5	6.8	1.4	86.2	4.1	
m-synephrine-d_3	0.3	2.0	13.2	80.7	3.8	
p-synephrine-d_3	0.3	1.0	1.8	93.8	3.2	

Other compounds were synthesized as previously described in the literature: *o*-octopamine (*13*), *o*-synephrine benzoate (-d_0 and methyl-d_3) (*14*), *o*-tyramine (*15*), *m*-octopamine-2,4,6-d_3 hydrochloride (*16*), *m*-synephrine-2,4,6-d_3 hydrochloride (*16*), *p*-synephrine (methyl-d_3) (*16*), *p*-octopamine-α,α'-d_2-3,5-d_2 hydrochloride (*16*), and *N*-methylphenylethanolamine (*17*).

The composition of the deuterated octopamines and synephrines was determined by NCI GC/MS of the corresponding PFP derivatives by using the molecular ions (m/z 591–596 for the octopamines and m/z 605–610 for the synephrines). These results are shown in Table I.

The deuterated standards contained less than 2% of the corresponding nondeuterated species. Each of the six deuterated standards in Table I was examined for possible contamination with the other five amines. Each isomer was found not to be contaminated with its other positional isomers, nor did any of the octopamines contain traces of the synephrines, or vice versa. The conditions of acid hydrolysis employed in the biological experiments were not found to cause a significant change in the deuterium distribution of the deuterated standards.

Instrumentation. This work was performed on a Hewlett-Packard 5985 GCMS system equipped with an EI/CI source with negative chemical ionization capability. The end of the fused silica capillary column ("ultrabond" cross-linked methylsilicone, 25 m × 0.2 mm i.d., 0.33 μm film; from Hewlett-Packard) was inserted into the source of the mass spectrometer where a pressure of 1 torr was maintained by adding methane. The GC injection port and interfacial region between the GC and the MS were maintained at 250 °C. The GC oven was maintained at 80 °C for 0.5 min following injection and then raised to 250 °C at a rate of 30 °C/min. The electron multiplier voltage was 2400 V; source temperature, 100 °C; electron beam current, 300 μA.

Extraction of Urine. A standard solution (100 μL, equivalent to 10 ng each of *o*- and *m*-octopamine-d_3 and 100 ng each of *p*-octopamine-d_4 and *o*-, *m*-, and *p*-synephrine-d_3) was added to a volume of urine containing 1 mg of creatinine in a disposable screw-capped tube. The pH of the mixture was adjusted to a value of 1 with concentrated HCl and the tube was placed in a water bath and maintained at 90 °C for 25 min. The urine was cooled, the pH was adjusted to a value of 6.0 with 2 N NaOH, and the liquid was placed on a column (5 × 0.8 cm) of AG 50W-X2 resin in a disposable pipet. The resin was washed with water (10 mL), sodium acetate (0.1 N, 25 mL), water (25 mL), and ethanol (70%, 1 mL), and the amines were eluted with 1 N ammonia in 65% ethanol (3 mL). The eluate was blown to dryness with a jet of nitrogen and the residue reconsituted in water (1 mL). The pH of the liquid was adjusted to a value of 6.0 with 0.5 N HCl and the resultant solution passed through a second column (1.5 × 0.8 cm) of AG 50W-X2 resin. The resin was washed with water (5 mL), sodium acetate (0.1 N, 10 mL), water (20 mL), and ethanol (70%, 1 mL). The amines were eluted with ammoniacal ethanol (1 mL) as before and the solvent removed under a stream of nitrogen.

Derivatization. Dried urinary extracts or standards were heated with PFPA (100 μL) for 15 min at 60 °C in a screw-capped vial. The PFPA was evaporated under a stream of nitrogen and the residue taken up in hexane.

Identification and Quantitative Determination of Amines. The 1:1 standard containing 1 ng/μL of each of the six octopamines and synephrines and their corresponding deuterated analogues was prepared daily and the amines were derivatized as above. The following ratios of intensities (measured by areas) were determined: $M^-/(M-HF)^-$ for the PFP derivatives of the nondeuterated compounds and $M^-_{nondeuterated}/M^-_{deuterated}$ (or, $M^-_{d_0}/M^-_{d_x}$). The corresponding ratios were determined for each derivatized biological extract (containing the internal standard) and compared to those obtained for the standard. When a 1:1 standard was subjected to the same procedure as the biological sample (acid hydrolysis, ion-exchange chromatography, GC/MS), there was no change in the $M^-/(M-HF)^-$ and $M^-_{d_0}/M^-_{d_x}$ ratios.

RESULTS AND DISCUSSION

Pentafluoropropionyl (PFP) derivatives of biogenic amines are widely used in GC analyses because they are easily formed and are stable for several hours. Although it has been suggested that other derivatives have advantages (*11*), the PFP derivatives have so far proven to be superior for NCI GC/MS analyses of the catecholamines (*18, 19*). Martin et al. (*18*) have investigated the fragmentation patterns of the PFP derivatives of dopamine, norepinephrine, epinephrine, and their *O*-methyl derivatives under NCI conditions. All of these compounds undergo dissociative resonance electron capture followed by cleavage of the benzylic C–O bond resulting in the formation of the *reagent-specific* ion ($C_2F_5CO_2$, m/z 163) as the base peak; however the molecular M^- and/or $(M-HF)^-$ ions are usually prominent also. We observed that the PFP derivatives of the octopamines and synephrines also exhibit relatively abundant M^- and $(M-HF)^-$ ions when subjected to NCI GC/MS (Table II).

The presence of appreciably intense M^- and $(M-HF)^-$ ions is important not only for sensitivity of the method but also

Table II. Kovats' Indexes and Intensities of Relevant Ions of PFP Derivatives of Some Biogenic Amines

PFP derivative	Kovats' index	mol wt	% total negative ion current		m/z
			M^-	$(M-HF)^-$	
phenylethanolamine	1325	429	0.7	3.8	409
o-octopamine	1365	591	22	18	571
o-tyramine	1370	429	0.0	31	409
N-methylphenylethanolamine	1385	443	5.9	9.9	423
m-octopamine	1430	591	18	12	571
m-tyramine	1435	429	0.4	27	409
o-synephrine	1440	605	3.0	4.0	585
p-tyramine	1463	429	0.1	5.5	409
p-octopamine	1465	591	38	2.7	571
m-synephrine	1470	605	6.0	10	585
norepinephrine	1490	753	33	6.0	733
p-synephrine	1520	605	10	7.0	585
epinephrine	1525	767	23	1.0	747
dopamine	1545	591	2.0	42	571
normetanephrine	1570	621	29	4.0	601
epinine	1590	605	1.0	0.1	585
3-methoxytyramine	1605	459	0.2	5.9	439
metanephrine	1620	635	17	13	615

for satisfying the criteria for identification of an unknown compound by GC/MS SIM. These criteria are (1) that at least two structure-specific ions (preferably one being the molecular ion) should be present, (2) that these two ions be present in the unknown sample at the same retention time as those of the authentic standard, and (3) that the ratio of the intensities of these ions derived from the biological sample should be identical with the corresponding ratio obtained from the authentic standard. Kovats' indexes and intensities of the M^- and $(M - HF)^-$ ions of the PFP derivatives of the octopamines, synephrines, and 12 other biogenic amines are shown in Table II.

The PFP derivatives of the three isomeric octopamines were completely resolved from one another as were the PFP derivatives of the three synephrines. The PFP derivatives of *p*-tyramine, *p*-octopamine, and *m*-synephrine have very similar retention times but they afforded M^- and $(M - HF)^-$ ions at different m/z values.

With our instrumentation, the limit of detection for an on-column injection was 100 fg for the molecular ion of *m*-octopamine–PFP which carries 18% of the negative ion current and 500 fg for the molecular ion of *m*-synephrine–PFP which carries 6% of the total ion current. Our results are very similar to those of Hunt and Crow who have reported that the molecular ion of the (pentafluorobenzylimine)trimethylsilyl derivative of dopamine carries 95% of the negative ion current and that the lower limit of detection for an on-column injection was 25 fg (*11*).

This remarkable sensitivity obtained with pure standards could not be achieved with the biological extracts employed in our experiments. This was because the finite population of electrons which are available in the ion source for ionization of the sample is seriously depleted by relatively small quantities of compounds with high electron affinities, thereby causing a sharp drop in sensitivity (*11*). We have found that the intensity of the negative ion signal becomes nonlinear with regard to concentration when the sample of *m*-synephrine–PFP exceeds 5 ng and that saturation of the negative ion signal occurs with sample size above 20 ng. Unfortunately, biological samples normally contain many compounds which can be derivatized by PFPA to form electron-capturing compounds. If these compounds are present in sufficiently large amounts, and if they elute shortly before (or simultaneously with) the compound of interest, a significant reduction in sensitivity ("quenching") will result. Thus investigations utilizing NCI GC/MS must usually be carried out with relatively small samples and the PFPA used for derivatization must be removed and replaced with hexane before the derivatized sample is injected into the system (*12*). Because of these saturation effects and the presence in samples of other electron-capturing ("quenching") substances, the practical lower limit of detection for the octopamines and synephrines in urine by NCI was found to be about 100 pg mg^{-1} creatinine.

Identification of Octopamines and Synephrines in Urine. Morning specimens of urine were obtained from five male and five female volunteers with ages ranging from 13 to 55. All the subjects were apparently healthy, were not receiving medication, and had abstained from citrus fruit and juice for 48 h prior to collection of the specimens.

In a typical analysis, an aqueous solution (100 μL) of the internal standard containing deuterated *o*-, *m*-, and *p*-octopamine (10 ng, 10 ng, 100 ng, respectively) and the deuterated synephrines (100 ng each) was added to urine (containing 1 mg of creatinine), which was then processed as described above. In each of the NCI GC/MS analyses depicted in Figures 1–3, the tracing at each m/z value was normalized to the highest peak. Figure 1A shows the PFP derivatives of deuterated and nondeuterated *p*-octopamine in the 1:1 standard mixture containing the 12 amines. Figure 1B is the corresponding tracing obtained from a derivatized urine extract. From Table III it is clear that the criteria (vide supra) for the unequivocal identification of *p*-octopamine are satisfied.

Figure 2 shows the results of the corresponding analyses of *o*- and *m*-octopamine–PFP by NCI GC/MS. Examination

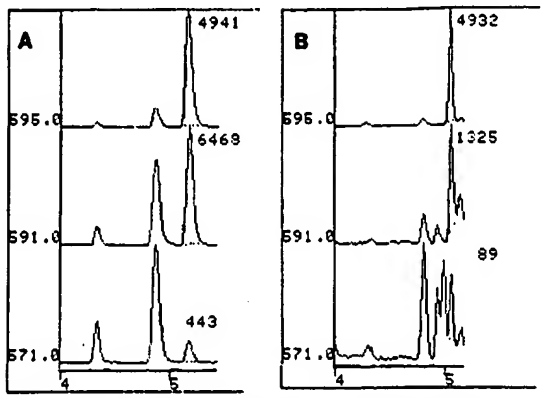

Figure 1. (A) The PFP derivatives of *p*-octopamine-d_0 and -d_4 in the 1:1 standard (corresponding to an on column injection of 250 pg) containing the 12 amines. (B) The corresponding derivatives in an aliquot (3 μL) of a derivatized urinary extract in hexane (25 μL) from urine containing 1 mg of creatinine and 100 ng of the *p*-octopamine-d_4.

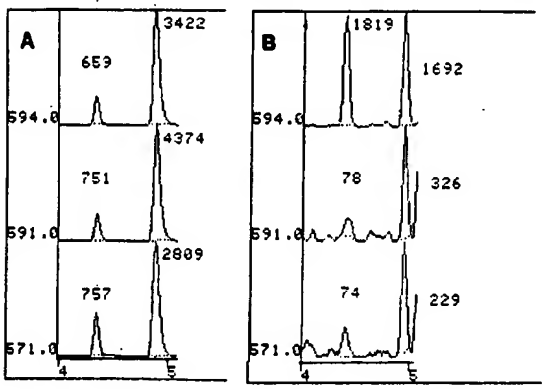

Figure 2. (A) The PFP derivatives of *o*- and *m*-octopamine-d_0 and -d_3 obtained by a repeat injection of the same amount of 1:1 standard shown in Figure 1. (B) The corresponding derivatives in an aliquot (3 μL) of a derivatized urinary extract in hexane (25 μL) from urine containing 1 mg of creatinine and 10 ng each of *o*- and *m*-octopamine-d_3.

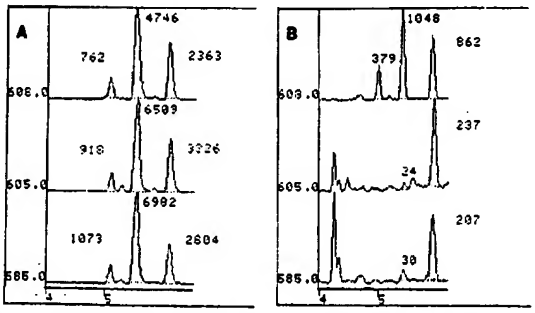

Figure 3. (A) The PFP derivatives of *o*-, *m*-, and *p*-synephrine-d_0 and -d_3 obtained by a repeat injection of the same amount of 1:1 standard shown in Figure 1. (B) The corresponding derivatives in an aliquot (3 μL) of a derivatized urinary extract in hexane (25 μL) from urine containing 1 mg of creatinine and 100 ng each of *o*-, *m*-, and *p*-synephrine-d_3.

Table III. Identification of Octopamines and Synephrines in Human Urine

PFP derivative	retention time, min		ratio of intensities of $M^-/(M - HF)^-$ ions	
	std	unknown	std	unknown
o-octopamine	4.2	4.2	0.99	1.05
m-octopamine	4.95	4.95	1.56	1.42
p-octopamine	5.30	5.30	14.6	14.9
o-synephrine	5.05		0.85	
m-synephrine	5.25	5.25	0.93	0.80
p-synephrine	5.65	5.65	1.19	1.14

Table IV. Recovery and Quenching of Deuterated Standards

deuterated amine	observed recovery, %	quenching, %
o-octopamine	11 (±5)	53 (±14)
m-octopamine	6 (±2)	59 (±16)
p-octopamine	4 (±2)	75 (±15)
o-synephrine	9 (±4)	61 (±11)
m-synephrine	6 (±2)	73 (±10)
p-synephrine	12 (±6)	46 (±14)

of Table III confirms unambiguously that both *o*- and *m*-octopamine are present in the urinary extract. This result was not an artifact arising from the presence of *o*-octopamine-d_0 in the internal standard (ratio of intensities, 0.001: Table I) since the corresponding ratio observed for the biological sample was 0.042.

Similarly Figure 3 was obtained from the analyses of the PFP derivatives of the isomeric synephrines by NCI GC/MS. *o*-Synephrine could not be detected in any urinary extract whereas *m*- and *p*-synephrine were both present (Table III). The ratio of intensities observed for *m*-synephrine ($M^-_{d_0}/M^-_{d_3}$, 0.023) is approximately seven times that expected from the contribution arising from the d_0 component of the internal standard (100 ng). When 10 ng of *m*-synephrine-d_3 was used as an internal standard instead of 100 ng, the anticipated 10-fold increase in this ratio was observed.

Quantitative Determination of Isomeric Octopamines and Synephrines. The amounts of *o*-, *m*- and *p*-octopamine and of *m*- and *p*-synephrine were determined by comparison of the ratio of the intensities of $M^-_{d_0}/M^-_{d_3}$ measured for the derivatized biological sample with that arising from the derivatized 1:1 standard and the results are summarized in Tables IV and V. All the analyses were carried out in duplicate and the percent mean variations were as follows: *o*-octopamine, 20%; *m*-octopamine, 16%; *p*-octopamine, 20%; *m*-synephrine, 24%; *p*-synephrine, 20%.

The recovery of each component was estimated by comparing the intensity of the molecular ion arising from 1 ng deuterated standard with that observed for a theoretically equal quantity in the urinary extract. Recoveries ranged from 4% to 12% in these analyses (Table IV) while the recoveries of similar amounts of internal standard from water were ca. 40%.

The quenching factor (46–75%, Table IV) was estimated in the following way. The derivatized urinary extract was coinjected with the derivatized internal standard, and the intensity of the M^- ion arising from the PFP derivatives of the deuterated amine was measured. This value was compared with the sum of the values arising from the separate injections of corresponding quantities of the derivatized urinary extract and derivatized internal standard. Consequently it may be seen that the apparent recovery of ca. 5–10% was caused in part by quenching due to coeluting components which have a high affinity for electrons: thus the actual recovery of these amines from urine is of the order of 10–20%.

Our choice of acid hydrolysis for deconjugation of the octopamines and synephrines was based on the following considerations: (1) In the human, endogenous *p*-octopamine and *p*-synephrine are excreted predominantly as acid-hydrolyzable conjugates. In the case of *p*-synephrine, this is neither a sulfate nor a glucuronide (*20*). (2) In the human, exogenous *m*-synephrine is excreted predominantly as the sulfate (oral, 80%; nasal 90%) with the remainder as a glucuronide, and both are released completely by acid hydrolysis (*21*). (3) In the human, exogenous (intravenous) *m*-octopamine is excreted free (50%) and as an acid-hydrolyzable conjugate (50%) (*22*). (4) Although nothing is known of the mode of excretion of *o*-octopamine and *o*-synephrine in the human, in the rat exogenous (intraperitoneal) *o*-octopamine is excreted free. *o*-Synephrine is excreted both free (50%) and as an acid-hydrolyzable conjugate (50%) (*7*) which is neither a sulfate nor a glucuronide. Extending the time of acid hydrolysis to 1 h at a higher temperature (boiling water bath) did not increase the observed amounts of the amines.

The ten samples of urine were also analyzed for unconjugated octopamines and synephrines by omitting the acidic hydrolysis (see Table V).

This is the first report of the presence of endogenous *o*-octopamine in a human biological fluid. The administration of *o*-octopamine to rats (*7*) resulted in the excretion of *o*-hydroxyphenylglycol (OHPG), *o*-hydroxymandelic acid (OHMA), and unchanged amine (ca. 66% as an acid-hydrolyzable conjugate). OHMA, in contrast to OHPG (*7*,

Table V. Urinary Concentration of Octopamines and Synephrines

subject (sex, age)	concentration, ng mg^{-1} creatinine											
	o-octopamine		*m*-octopamine		*p*-octopamine		*o*-synephrine		*m*-synephrine		*p*-synephrine	
	free	total	free	total	free	total	free	total	free	total	free	total
SE (F, 19)	0.6	0.7	1.0	1.7	1.5	33	ND[a]	ND	2.0	2.8	1.0	25
KT (F, 25)	0.3	0.7	1.7	3.4	1.2	32	ND	ND	2.0	2.3	1.9	17
LB (F, 35)	0.2	0.2	0.8	0.8	0.3	6	ND	ND	1.1	0.8	1.2	41
MC (F, 42)	0.5	0.7	1.5	2.0	1.6	61	ND	ND	1.0	0.7	1.9	9
JR (F, 55)	0.1	0.6	1.1	2.2	0.8	7	ND	ND	2.4	2.7	2.5	11
LC (M, 13)	0.4	0.5	1.6	3.1	0.8	9	ND	ND	1.4	1.6	2.5	8
SR (M, 29)	0.4	0.5	1.3	2.6	0.8	12	ND	ND	1.4	1.8	1.2	8
KI (M, 33)	0.4	1.0	1.3	2.1	2.2	49	ND	ND	1.9	2.3	1.4	13
LC (M, 42)	0.3	0.6	0.6	1.2	1.5	28	ND	ND	1.0	1.3	4.0	20
CW (M, 55)	0.3	0.7	0.7	1.6	0.9	10	ND	ND	1.5	2.1	0.4	12
mean	0.3	0.6	1.0	2.1	0.9	25	ND	ND	1.6	1.8	1.8	16
std dev	0.1	0.2	0.5	0.8	1.2	19			0.5	0.7	1.0	10

[a] ND: not detected (<100 pg mg^{-1} creatinine).

23), is a normal constituent of rat (7) and human (24) urine and is found in elevated quantities in the urine of patients with phenylketonuria (14). However, it was not possible to detect endogenous *o*-octopamine in the urine of normal humans (7) and patients with phenylketonuria (14) by electron-impact GC/MS methods. The present report confirms our earlier proposal (7, 14) that urinary OHMA originates predominantly (if not entirely) from *o*-octopamine and that the latter is a naturally occurring (i.e., biogenic) amine.

It has been established that *m*-octopamine is present in rat salivary gland (3, 8), rat and bovine adrenal glands (8), and rat brain (4). However, this constitutes the first account of its occurrence in a biological fluid. Exogenous *m*-octopamine is metabolized by mammals to *m*-hydroxyphenylglycol (25) and *m*-hydroxymandelic acid (22, 25) which both occur naturally in human and rat urine (23). However, part of the administered dose is not deaminated (22, 25) and our finding that *m*-octopamine is excreted as an acid-hydrolyzable conjugate and as the unconjugated amine (in approximately equal quantities) agrees with that of Hengstmann et al. (22) who administered *m*-octopamine intravenously to humans.

Unconjugated *p*-octopamine was identified first in human urine by two-dimensional paper chromatography in amounts of 0.5 ng mg^{-1} creatinine (26) and as an acid-hydrolyzable conjugate in amounts of 0–20 ng mg^{-1} creatine (20). The values reported here are in excellent agreement with these earlier estimates.

o-Octopamine is a poor substrate for norepinephrine *N*-methyltransferase (6) and James et al. found that *o*-synephrine could not be detected in urine after the administration of 250 μg of *o*-octopamine to rats (7). Therefore *o*-octopamine may not be a precursor of *o*-synephrine which may account for our failure to detect *o*-synephrine. However, it should be borne in mind that the lower limit of detection of *o*-synephrine by our method is 100 pg mg^{-1} creatinine and that *o*-synephrine might be present in concentrations lower than this limit.

Although *m*-synephrine has been found to occur naturally in adrenal gland (9, 10) this is the first report of its natural occurrence in mammalian urine and our results indicate that endogenous *m*-synephrine is present entirely in the unconjugated state. This concurs with the results observed (27) when *m*-synephrine was administered intravenously to humans: 73% of the excreted amine was unconjugated. In contrast, when administered orally, a substantial proportion (50–60%) of the dose was not deaminated and was excreted almost entirely in the conjugated form (21, 27).

p-Synephrine was identified in human urine by chromatographic means some 20 years ago (20, 28). It is excreted as an acid-hydrolyzable conjugate in amounts which vary markedly from individual to individual (e.g., 0–1600 ng mg^{-1} creatinine) (20). These observations, together with the large variation in the daily excretion of *p*-synephrine by a given individual, suggested that urinary *p*-synephrine was primarily of dietary origin. Subsequently, it was discovered that *p*-synephrine (29) and *p*-octopamine (30) were present in citrus fruits in appreciable quantities (e.g., *p*-synephrine, orange juice, 4.8 μg mL^{-1}). Consequently we have analyzed the juice from fresh citrus fruit for the octopamines and synephrines in the manner described above. Unconjugated *p*-octopamine was present in orange (160 ng mL^{-1}), grapefruit (9 ng mL^{-1}), and lemon (0.8 ng mL^{-1}) while orange and grapefruit juice contained 16 μg mL^{-1} and 8 ng mL^{-1}, respectively, of unconjugated *p*-synephrine. The other isomeric octopamines and synephrines were absent (lower limit of detection, 100 pg mL^{-1}). When ten human subjects abstained from citrus fruit or juice for 48 h prior to donation of specimens, we observed that *p*-synephrine was excreted (predominantly as an acid-hydrolyzable conjugate) in amounts varying from 8 to 41 ng mg^{-1} creatinine.

These results demonstrate the utility of the new capillary column electron capture NCI GC/MS assay for isomeric octopamines and synephrines in urine. The high sensitivity of this technique has made possible the detection, for the first time, of *o*- and *m*-octopamine and *m*-synephrine in human specimens. Research, including the development of tandem mass spectrometric methods (MS/MS), is continuing in this area.

ACKNOWLEDGMENT

This work was supported by the Medical Research Service of the Veterans Administration.

Registry No. *o*-Octopamine, 2234-25-5; *m*-octopamine, 536-21-0; *p*-octopamine, 104-14-3; *o*-synephrine, 575-81-5; *m*-synephrine, 532-38-7; *p*-synephrine, 94-07-5; *p*-octopamine PFP deriv., 62237-94-9; *p*-octopamine-d_4 PFP deriv., 90320-70-0; *o*-octopamine PFP deriv., 90320-71-1; *o*-octopamine-d_3 PFP deriv., 90320-72-2; *m*-octopamine PFP deriv., 77745-52-9; *m*-octopamine-d_3 PFP deriv., 90320-73-3; *o*-synephrine PFP deriv., 90320-74-4; *m*-synephrine PFP deriv., 90320-75-5; *p*-synephrine PFP deriv., 77862-54-5; *o*-synephrine-d_3 PFP deriv., 90320-76-6; *m*-synephrine-d_3 PFP deriv., 90320-77-7; *p*-synephrine-d_3 PFP deriv., 90320-78-8.

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RECEIVED for review February 6, 1984. Accepted April 2, 1984.

Exhibit C

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 239, No. 3, March 1964
Printed in U.S.A.

The Isolation and Identification of *l*-Synephrine in the Leaves and Fruit of Citrus*

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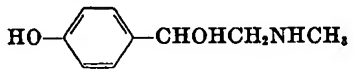
(Received for publication, June 17, 1963)

During a study of the relationship between trace element deficiencies and the free amino acids in citrus, an unknown basic nitrogen compound was detected in leaf extracts. In most samples there was more of the unknown constituent than of any other basic nitrogen compound. However, it was not present in leaf samples from trees with manganese deficiency. A comparison of its paper chromatographic R_F values with those of many known materials suggested that it was a compound not commonly found in plants. After extraction and isolation, it was identified by degradation studies and infrared analyses as *l*-*p*-hydroxy-α-(methylaminomethyl)benzyl alcohol, (synephrine, Sympatol),



an *Ephedra* alkaloid not previously known to occur in plants.

EXPERIMENTAL PROCEDURE

Extraction and Separation—Leaves collected from tangerine trees were frozen and extracted in a Lourdes mixer with either 80% aqueous ethanol or anhydrous methanol, the latter being a more suitable solvent. The extract was adjusted to pH 8.5 with ammonium hydroxide and passed through a Dowex 50-X4 ion exchange column. The column had first been treated with 1 N sodium hydroxide, followed by 4% hydrochloric acid, again with sodium hydroxide, and finally with water until the pH of the effluent was between 8 and 9. After passage of the extract, the column was washed with water and then with methanol. The *l*-synephrine was then eluted with 2 N ammonium hydroxide in methanol. The eluate was taken to dryness in a rotary vacuum evaporator at 50°. *l*-Synephrine crystals formed during the final stages of evaporation. These were dissolved in a small volume of hot methanol and recrystallized at 4°. This material could also be crystallized from water or from 80% acetone and water.

IDENTIFICATION

Properties of Base—The crystals were clear plates, soluble in methanol and water but only slightly soluble in acetone, ether, or the higher alcohols. The melting point is a decomposition point and is not a good criterion of purity since it varies with the rate of heating. Decomposition of *l*-synephrine, heated at approximately 1° per minute, took place at 162–164°.

$C_9H_{13}NO_2$

Calculated: C 64.65, H 7.84, N 8.38
Found: C 64.58, H 7.80, N 8.14

The synephrine extracted from citrus leaves was the levo isomer with an optical rotation in 0.5 N HCl, $[\alpha]_D^{25} = -55.60°$.

Derivatives

Oxalate Salt—This salt was readily prepared by adding slowly 159 mg (0.0018 mole) of dry oxalic acid to 400 mg (0.0024 mole) of synephrine in 25 ml of boiling methanol. Small crystals formed immediately, and after cooling, they were collected on a filter. When the salt was made from pure synephrine, an 86% yield was obtained. The oxalate salt was a stable derivative and was useful in the purification of *l*-synephrine. For purification, the salt was suspended in boiling methanol, and while the solution was still heating, water was added slowly until the crystals dissolved. A small amount of Darco G-60 activated carbon was added, and the hot mixture was filtered. Large, clear, thin plates formed when the filtrate was cooled to −18°. Infrared curves and elemental analysis before and after regeneration from this salt showed that the synephrine had not been altered. Elemental analysis of the salt suggested that a mole for mole combination did not take place but rather 2 moles of the base combined with 1 mole of the acid.

$(C_9H_{13}NO_2)_2(C_2H_2O_4)$

Calculated: C 56.32, H 7.09, O 30.01, N 6.57
Found: C 56.29, H 6.71, O 30.00, N 6.82

The melting point of the crystals was 221–222° (decomposition). Further evidence for the composition of the oxalate salt was obtained as follows. A minimal amount of 0.5 N NaOH was used to dissolve 500 mg of synephrine oxalate. Approximately 8 volumes of methanol were added, and a precipitate was formed. After settling overnight in a refrigerator, the precipitate was filtered and washed with methyl alcohol. It had a neutral reaction and was identified as sodium oxalate by infrared analysis. This decomposition yielded 132 mg of sodium oxalate, which is the approximate theoretical yield. The free base was recovered by adjusting the filtrate to pH 8.5 and passing it through a Dowex 50-X4 ion exchange column which had been prepared as described in "Extraction and Separation."

Degradations

Alkaline Fusion—The unknown was mixed with an excess of dry KOH in a tube and heated with an open flame in an atmosphere of nitrogen. A volatile amine distilled and was collected in a methanol solution of picric acid. A crystalline picrate salt was thus obtained which proved to have the same infrared absorption spectrum as an authentic sample of methylamine picrate.

* Florida Agricultural Experiment Stations Journal Series No. 1681.

The residue from the fusion was made acid with hydrochloric acid, taken to dryness, and then triturated with methanol. After concentration, a crystalline product was isolated which was found to have an infrared absorption spectrum identical with that of an authentic sample of *p*-hydroxybenzoic acid.

Hofmann Degradation—Proof that the nitrogen atom was not in a ring was obtained by methylation of the base with methyl iodide. The resulting salt, m.p. 250° (decomposition), was subjected to Hofmann degradation by heating it with 30% aqueous sodium hydroxide. A volatile amine was collected and identified by infrared analysis as trimethylamine by preparation of its picrate salt.

Tests for Amines

A Van Slyke determination indicated that the unknown contained no significant amount of primary amino nitrogen. A test for tertiary amines which involved heating the unknown with acetic anhydride and citric acid, according to the method of Feigl (1), was negative. A positive test was obtained for a secondary amine, when the unknown was treated with 5% basic copper sulfate, and extracted with carbon disulfide and benzene (1).

Infrared Studies

Absorption curves were made with a Beckman model IR-4 double beam instrument with 1.5 mg of sample in a KBr pellet. From the results of the degradation studies, the tests for amines, the elemental analyses, and the negative tests for alkyl ether groups, it was considered likely that the unknown was *l*-synephrine. A synthetic sample of racemic synephrine tartrate was obtained, and the free base was regenerated from this salt. The infrared absorption curves obtained for racemic synephrine and the unknown were quite different, especially in the 3- to 4-μ and the 9- to 11-μ regions. There were also pronounced shifts in the 7- to 9-μ region (Fig. 1). However, when the oxalates were compared, the main differences were in the region from 11.8 to 12.8 μ, and these were primarily shifts. The tartrates also showed only slight differences. Purification procedures did not greatly change the differences in absorption peaks. These differences were suspected to be due to different crystal structures in the racemic and levo forms. Attempts to resolve the racemic form either as the tartrate, malate, or quinate or with amino acid oxidases were unsuccessful. Therefore, the unknown was racemized by refluxing a sample for 24 hours in an aqueous solution made slightly acid, approximately pH 1 to 2, with hydrochloric acid. The recovered base then gave an infrared curve identical with that of the racemic sample. The optical rotation of the racemized natural product was $[\alpha]_D^{25} = +5.80°$. The infrared absorption curves of the two racemic oxalates were also found to be identical.

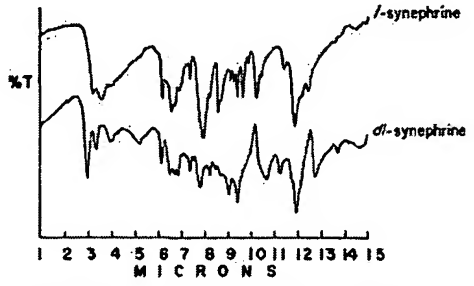


FIG. 1. Infrared absorption curves of *l*-synephrine and *dl*-synephrine.

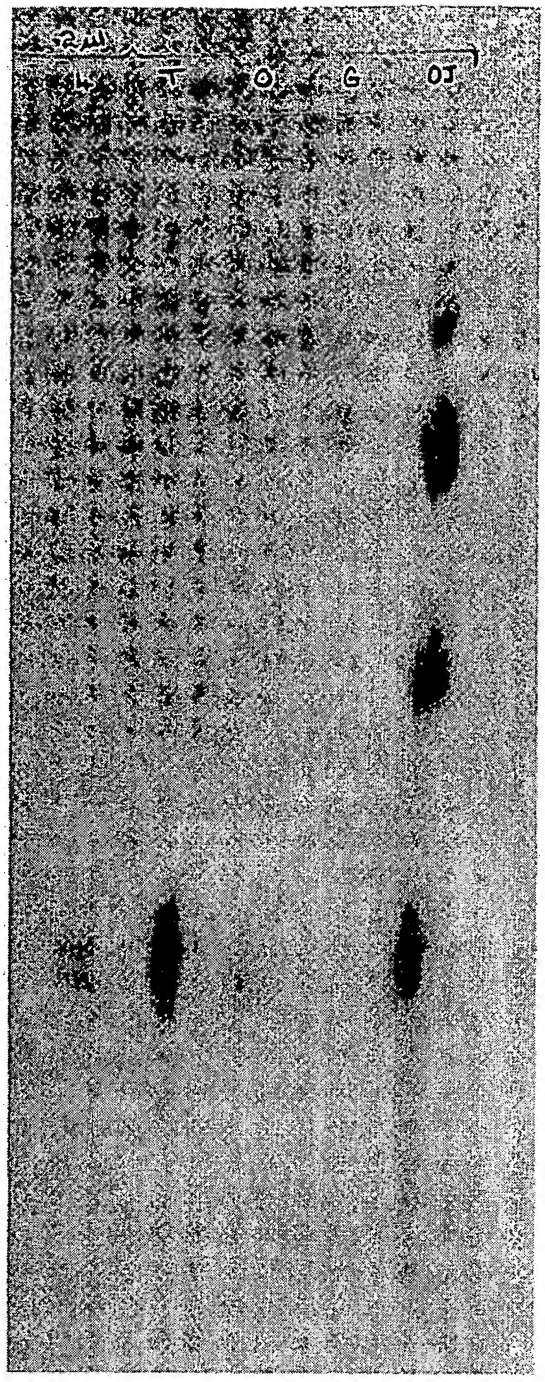


FIG. 2. Paper chromatogram showing synephrine in: *4*, known sample; *T*, tangerine leaves; *O*, orange leaves; *G*, grapefruit leaves; and *OJ*, orange juice. Spots in descending order are lysine, arginine, histidine, and synephrine.

Finally, synthetic samples of *l*-synephrine were obtained, and their infrared absorption curves were found to be identical in all respects with those run on samples extracted from citrus.

Chromatography

The best means found for separating *l*-synephrine in plant samples was by chromatography on EDTA-buffered paper with phenol-cresol as a solvent (Fig. 2) (2). By this procedure, synephrine has an R_F of 0.67. When a known sample of synephrine was compared with the unknown, identical R_F values were obtained. This was also true when two other solvent combinations were used.

OCCURRENCE

l-Synephrine has been observed in several hundred samples of citrus leaves with the greatest amounts occurring in mandarin varieties. Sufficient quantitative determinations have not been made to establish the range of concentration. Single analyses on tangerine and orange leaves showed them to contain 3.1 and 0.5 mg per g (green weight), respectively. Up to this time, none has been found in the leaves of grapefruit, pumelo, or shaddock (Fig. 2). However, in tangelos, which are hybrids of mandarin-pumelo crosses, the alkaloid is present. *l*-Synephrine has not been found in the roots of any variety of citrus. One sample of Valencia orange juice from a commercial extractor contained 4.8 mg per liter. Further studies will be reported elsewhere on the range of concentration in which synephrine occurs in citrus fruit juices.

DISCUSSION

Synephrine was first reported as a synthetic compound by Legerlotz (3) in Germany in 1927. It is used as a sympathomimetic agent, as a vasopressor in hypotension (4), and as an antihistamine in the treatment of common colds (5). Recently, synephrine has been reported to occur in human urine (6–8). The amounts present in urine fluctuate considerably, which led Kakimoto and Armstrong (7) to suggest that, rather than being a normal metabolite, synephrine probably comes from dietary sources. The presence of *l*-synephrine in citrus would tend to further substantiate this. Recently, Axelrod (9) demonstrated that a nonspecific enzyme in rabbit liver would form adrenaline from synephrine. This would suggest that citrus juice may be an indirect dietary source of adrenaline in man.

l-Synephrine is the only alkaloid known to occur in citrus. However, other nitrogen bases have been reported, and these have been reviewed by Sinclair (10). According to this review, betaine and stachydrine were isolated by the Japanese worker, Yoshima, in 1918. These are the methylated derivatives of glycine and proline, respectively. Choline was reported as a constituent of the juice in 1935 by Nelson *et al.* and later confirmed by Swift and Veldhuis. Putrescine was found by Herbst and Swell. The lack of structural similarity between synephrine and these bases, known to occur in citrus, makes it unlikely that their biosyntheses are related. Pisano *et al.* (6) have speculated that the biosynthesis of synephrine in animals may follow the route of β-hydroxylation of tyramine to form norsynephrine followed by the N-methylation of norsynephrine to synephrine by the same enzyme which converts norepinephrine to epinephrine. It will be interesting to determine if the same pathway is followed in plants.

SUMMARY

l-*p*-Hydroxy-α-(methylaminomethyl)benzyl alcohol (synephrine, Sympatol) has been isolated and identified from citrus leaves and juice. Identification was by means of degradations, derivatives, chromatography, and comparison of infrared absorption curves with those of samples from synthetic origin. Synephrine is not previously known to occur in plants.

Acknowledgments—We wish to express our thanks to Dr. R. O. Clinton of the Sterling-Winthrop Research Institute for a sample of *l*-Sympatol hydrochloride, and to C. H. Boehringer, Sohn Ingelheim am Rhein for a generous supply of the free base of *l*-Sympatol. Appreciation is also expressed to Mrs. Phyllis Farquhar for doing much of the chromatography in this study.

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